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RESEARCH ARTICLE OPEN ACCESS

EXTRACTION OF CHLORPYRIPHOS FROMA COMMERCIAL FORMULATION AND BIOLOGICAL SAMPLE

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ABSTRACT

Modern analytical methods for extracting pesticide are found in the literature, but the required equipment not always available. Given the large human, animal and environmental exposure to pesticides, and the inaccessibility to analytical methods, it is important to rescue simple methods for identifying pesticides. This research aimed to evaluate the efficiency of extracting an agrochemical active from a commercial formulation, directing its application in emergencies by cost, quality, and speed. Rapid extraction using three steps comprises: sample purification, extraction using only one solvent and identification by Thin Layer Chromatography (TLC). The determination of enzymatic cholinesterase was carried out, in triplicate, to control the action of the pesticide. The entire study was compared to a chlorpyrifos purchased from Sigma. The same procedure was performed with serum from rats contaminated with chlorpyrifos collected 3 h after administration. TLC provides characteristics of the active extracted as chlorpyrifos from Sigma, even using biological sample as serum (traces), while the inhibition of cholinesterase for the concentrations tested in comparison with the control, were statistically significant, respectively to that extracted and Sigma. The procedures applied were adequate to isolate the active, confirmed by TLC and by the cholinesterase inhibitory capacity.

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INTRODUCTION

Due to the high toxicity of organochlorines, organophosphate compounds emerged between 1945 and 1970 (FAO, 1997) and still remain in use. One of the commonly used organophosphatesis Chlorpyrifos [O,O-diethyl O-3,5,6-trichloropyridin-2-ol phosphorothioate] which was registered in 1965 (Michigan State University, 1993). Organophosphates are responsible for more than 36% of the total global chemical pesticide market.They are the most used substances in the control of agricultural pests, due to them being highly effective in controlling sucking and chewing insects, ensuring high agricultural production (SHAPIRO *et al*., 2016; ZHANG *et al*., 2014). Organophosphate compounds have at least one P-C bond (phosphorus-carbon) similar to carbon–carbon (C–C), which is very resistant to oxidation and hydrolysis (DOS SANTOS *et al*., 2007).

Although they are derived from elemental phosphorus, mainly from the P4 allotrope, they cannot be synthesized directly from the phosphorus element, as it is trivalent in its natural form (P^{III}).In order to synthesize an organophosphate compound, the P element must be pentavalent (P^{V}) (DOS SANTOS *et al*., 2007). They are therefore obtained from elements such as phosphorus trichloride (PCl_3), trialkyl phosphite ($P(OR)_3$), phosphine (PH_3), and sodium hypophosphite ($NaPO_2H_2$) (DOS SANTOS *et al*., 2007; WEFERLING; ZHANG; CHIANG, 2016). In most cases, the insecticidal activity of the organophosphate occurs due to its neurotoxic action through the inhibition of the enzyme acetylcholinesterase (AChE).This enzyme is responsible for the hydrolysis of acetylcholine (ACh), the main neurotransmitter in the central and peripheral nervous systems (CAVALCANTI *et al*., 2016b). Inhibition of AChE induces changes in the active site of the enzyme, causing the accumulation of ACh in synaptic clefts resulting in cholinergic hyperstimulation leading to insect death (CAVALCANTI *et al*., 2016b; MRDAKOVIĆ *et al*.,

2016). Among the organophosphate compounds, some are directly biologically active (phosphates) (Figure 1-I), whereas others need biotransformation to be biologically active(phosphorothioates) (Figure 1-II), with their toxic form being the "oxon" analogs (VALE, 1998). Chlorpyrifos belongs to the phosphorothioate group because it contains a sulfur atom (Figure 2) and therefore needs to be metabolized to be biologically active (TIMCHALK *et al*., 2002; VALE, 1998).

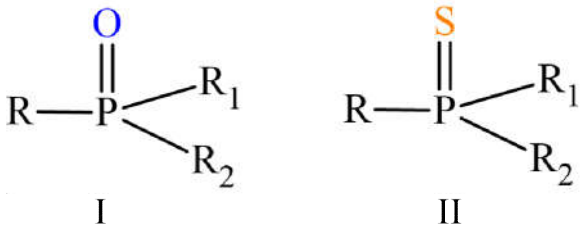

Figure 1. General structures of organophosphate compounds. I: phosphate group; II: phosphorothioate group

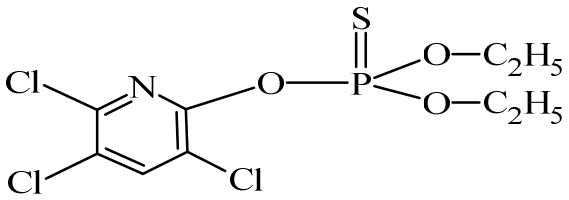

Figure 2. Chemical structure of chlorpyriphos

Metabolism of Chlorpyrifos occurs through cytochrome P450 (CYP) present in the liver, wherein Chlorpyrifos is transformed intochlorpyriphos-oxon [diethyl 3,5,6-trichloropyridin-2-yl phosphate], which istoxic for organisms and is highly inhibitory for the enzyme AChE (ALVARENGA, 2013). To transform into its toxic form, chlorpyrifos first comes into contact with cytochrome P450 to obtain an intermediate compound, fofoxityran, which is further desulfurized (replacingS for O) to form chlorpyrifos-oxon, as shown in Figure 3 (ALVARENGA, 2013; TANG *et al*., 2001). Organophosphate poisoning is an imminent risk for humans, farms and domestic animals (BERTONI *et al*., 2017; GOMES *et al*., 2020; GRECCO *et al*., 2009; MELO; OLIVEIRA; LAGO, 2002; OLIVEIRA-FILHO *et al*., 2010), but the risk to humans is substantially more severe.

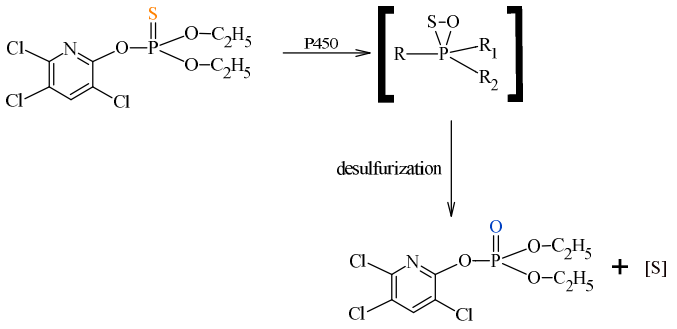

Figure 3. Biotransformation of chlorpyriphos by cytochrome P450. I: chlorpyriphos; II:fofoxityran; III – chlorpyriphos-oxon

Notably, the risk of suicide and death from this mode of intoxication is high. According to Silva and Garrido (2021), the use of pesticides for self-intoxication and suicide resulted in approximately 110,000 deaths annually between 2010–14.This number is equivalent to 13.7% of the total number of suicides that took place worldwide. Self-intoxication remains a large-scale public health challenge, accounting for at least one in seven suicides (MEW *et al*., 2017). In Brazil, the number of cases of organophosphate poisoning reached 62,000 between 1999 and 2009, with 5,600 cases per year and approximately 1,870 deaths, andthe southern and southeastern regions being most severely affected (CAVALCANTI *et al*., 2016a). For an assertive diagnosis of organophosphate poisoning, laboratory tests, such as blood count, ionograms, liver function markers, cardiac markers, and blood gases can be applied. However, the most effective method is the determination of erythrocyte cholinesterase (AChE) and plasma cholinesterase (BChE), which are the main markers in cases of intoxication (PEARSON *et al*., 2017; SILVA; GARRIDO, 2021). At the therapeutic level, drugs such as activated charcoal, muscarinic agents such as atropine, oximes, anticonvulsant/neuroprotective drugs are currently used. However, the use of bioscavengers, which are enzymes capable of effectively catalyzing nervous agents, is currently under discussion for treatment of either acute or chronic cases (SILVA; GARRIDO, 2021). Therefore, we investigated the inhibitory ability of chlorpyriphos inthe biotransformation processof living organisms. We also examined how to obtain the active compound from a commercial formulation known as Pyrinex, aiming to help in clinical analysis of chlopyriphosfrom cases of intoxication.

MATERIAL AND METHODS

Material: Commercial Pyrinex 480 EC (Adama®, Makhteshim Chemical Works Ltd, Israel) was kindly donated by the farmers of Ibiúna city (SP, Brazil), D.S.Silva, and D.S.Marçal. Chlopyriphos (code 45.395–100 mg) was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Additional materials used were hexane, acetic acid (Anidrol, Diadema, Brazil), acetone (F. Maia, BH, Brazil), bromophenol blue, petroleum ether (Synth, Diadema, SP, Brazil), sodium sulfate (Ecibra, Santo Amaro, SP, Brazil), silver nitrate (Sinergia, Campinas, Brazil), and palladium chloride (Dinâmica, Indaiatuba, SP, Brazil).

Extraction of organophosphate active from commercial Pyrinex 480 EC: Three systems were used to work with 135 mL of Pyrinex, using the same procedure simultaneously (Figure 4). In each system, 45 mL of Pyrinex was placed in a round-bottomed flask containing a few boiling chips to prevent bumping. Acetone (20 mL) was added to the flask to act as a purifier and to clean up the commercial formulation through its ability to extract common ingredients such as resins, sulfur, mineral oils, waxes, pine tar, dyestuffs, organic accelerators, antioxidants, and peptizing agents (CHANDRASEKARAN, 2007). Moreover, acetone was also used with the purpose of extracting both nonpolar (organochlorines) and polar (organophosphorus) pesticides (LUKE; FROBERG; MASUMOTO, 1975). A reflux condenser was coupled witha flask and then heated using a heating mantle for 20 minutes after reaching boiling point. After cooling the samples to room temperature, they weretransferred to a separation funnel. Next, 20 mL of 2% sodium sulfate (Ecibra®, Santo Amaro, SP, Brazil) was added at the first extraction step, with petroleum ether used to promote solvent exchange in a salting-out effect, which relies on changes in solubility based on ionic strength (LUKE; FROBERG; MASUMOTO, 1975; ZHANG; WONG, 2011).The petroleum ether extraction was repeated twice. Finally, the total extract was placed on an electric hot plate until it was dry to obtain a white solid with a yield of 52% (33.41 g).

Thin Layer Chromatography (TLC): The advantages of TLC are its speed, low cost, and efficiency of separation and identification (NEICHEVA; KOVACHEVA; KARAGEORGIEV, 1990). The dry residue was dissolved in petroleum ether (0.5 mL) and spots were applied onto an aluminum oxide layer chromatographic plate precoated with silica gel GF 254 (TLC AlugramXtra Silica Gel 60, Macherey-Nagel, Germany).Alternatively, chromatography paper (1Chr, Whatman International Ltd, Maidstone, England) can replace TLC. The mobile phase used was hexane:acetone (8:2, v/v). The end plates were dried and detected using UV irradiation and were sprayed with a solution of bromophenol blue as the developer (0.05 g) in acetone (10 mL).They were then filled to 100 mL with 1% $AgNO_3$ in 1:3 v/v acetone:water), and dried at 80 °C for 10 min in an oven. Presence of blue color indicates the presence of organophosphate.

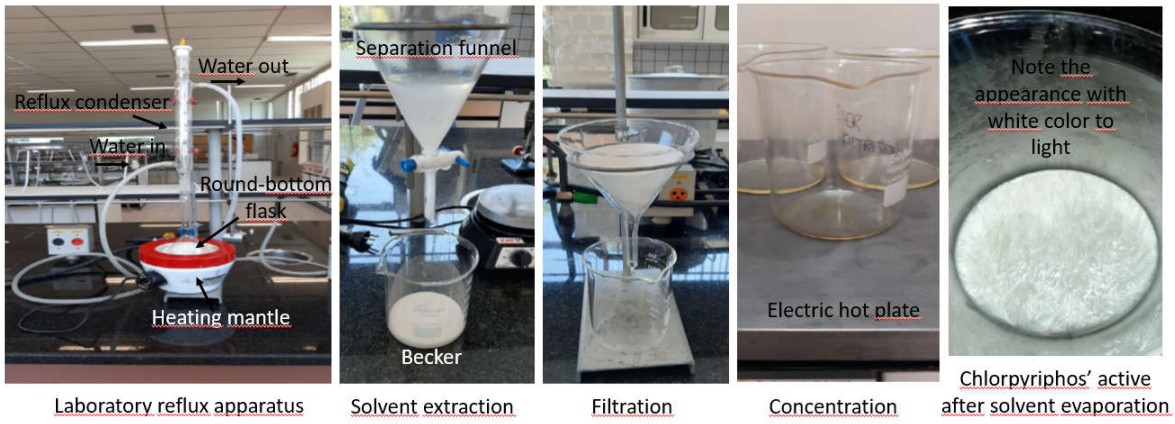

Figure 4. Method for extracting pesticide (chlorpyriphos) active from commercial formulation (Pyrinex 480 EC) using simple laboratory apparatus and steps: clean up, extraction, filtration, and concentration. Note the outcome after solvent evaporation to obtain a white color solid representative of chlorpyriphos

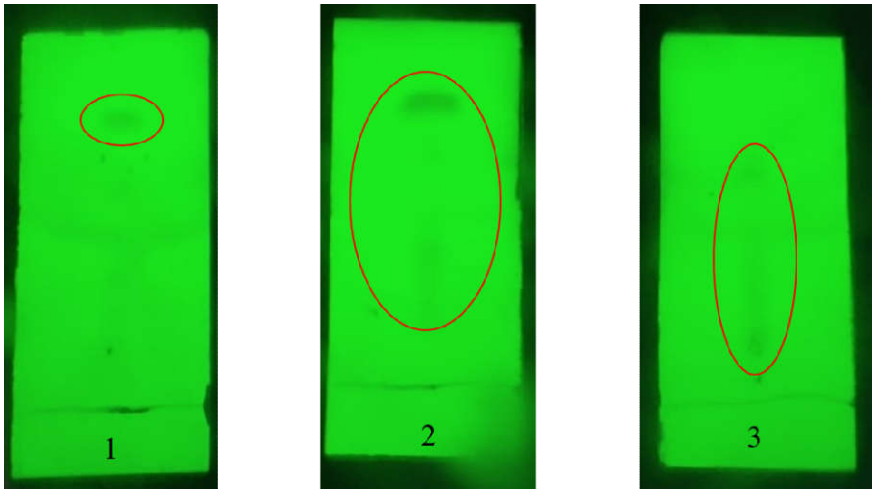

Figure 5. Thin Laver Chromatography. 1: Sigma® Chlorpyriphos Standard; 2: Material extracted from commercial sample Pyrinex ® 480 EC; 3: Biological material (animal serum). Plates are exposed to long-wave UV rays. Note the subtle trace in 3, which is due to the low concentration of chlorpyriphos in the serum samples

The plate was sprayed with 5% acetic acid to remove the background color, which then changed to a brown color.

Inhibitory ability of cholinesterase enzyme

In vitro: The activity of cholinesterase was determined using a commercial kit from Bioclin® (Quibasa, QuimicaBásicaLtda, Belo Horizonte, MG, Brazil), as described previously (WERNER *et al*., 2015). The BioControl N (Bioclin®)is a normal human serum calibrator which served as negative control in a matrix for fortifying three different concentrations of extracted chlorpyriphos (4.4, 5.4, and 6.4 mg/mL).This was compared to the resultsobtained using commercialchlorpyriphos from Sigma®. The concentrations of pesticides selected were based on those described by Demir *et al*. (2011) and Hallal *et al*. (2019). Dimethyl sulfoxide (DMSO)was used as a solvent to solubilize the pesticide and was analyzed to determine its influence on the enzymatic reaction. The cholinesterase inhibitor, neostigmine (Sigma®), was used as a positive control (WERNER *et al*., 2015). All assays were performed in triplicate.

In vivo: Bloodwas collectedfrom Wistar malerats (n=3) which were purchased from Animais de Laboratório (Anilab, Paulínia, SP, Brazil). They were orally administered with 15 mg/kg of chlopyriphos from Sigmavia gavage feeding. Serum cholinesterase was determined using the same procedure described above. The use of animals was approved by the Committee for the Care and Use of Experimental Animals at the University of Sorocaba (CEUA 174/2020).

Statistical Analysis

Results [mean± standard error of mean (S.E.M)] were statistically analyzed by one-way analysis of variance (ANOVA) with the confidence level set at 5% ($p<0.05$).

RESULTS AND DISCUSSION

The extraction procedureforPyrinex® 480 EC was followed in a simple way as described by Luke, Froberg, and Masumoto (1975). This resulted in a volume of approximately 135 ml, containing 33.41 g of active principle, which was equivalent to 52% yield.This process is highly efficient owing to the solvents used. The same procedure was appliedto biological material (animal serum samples), resulting in a mass of 0.006 mg (0.04% of the initial mass) of pesticide, due to the rapid metabolization of chlorpyrifos (MARTY *et al*., 2012; TANVIR *et al*., 2016). Another factor that can influence the small amount of recovered pesticide is the amount of lipids and other components that can influence the cleaning and analysis of the sample (WILLIAMS *et al*., 2011). TLC was performed to confirm the type of material obtained, in comparison with the Sigma® chlorpyriphos standard (Figures 5 and 6). This chromatographic method is effective for comparison and assertion that the desired compounds have been accurately recovered. During the measurement of plasma cholinesterase, a complementation was carried out, because no direct inhibition of the target enzyme by chlorpyrifos pesticide was found in the literature.

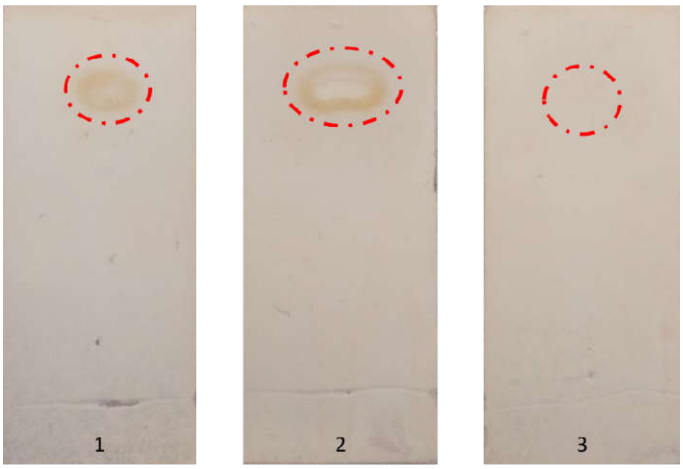

Figure 6. Thin Laver Chromatography. 1: Sigma® Chlorpyriphos Standard; 2: Material extracted from commercial sample Pyrinex ® 480 EC; 3: Biological material (animal serum). Chromatographic plates were sprayed with 0.5% paladium chloride in 2N HCl. Here, the chlorpyriphos sample trace/halo is very subtle, but it is noticed in 3

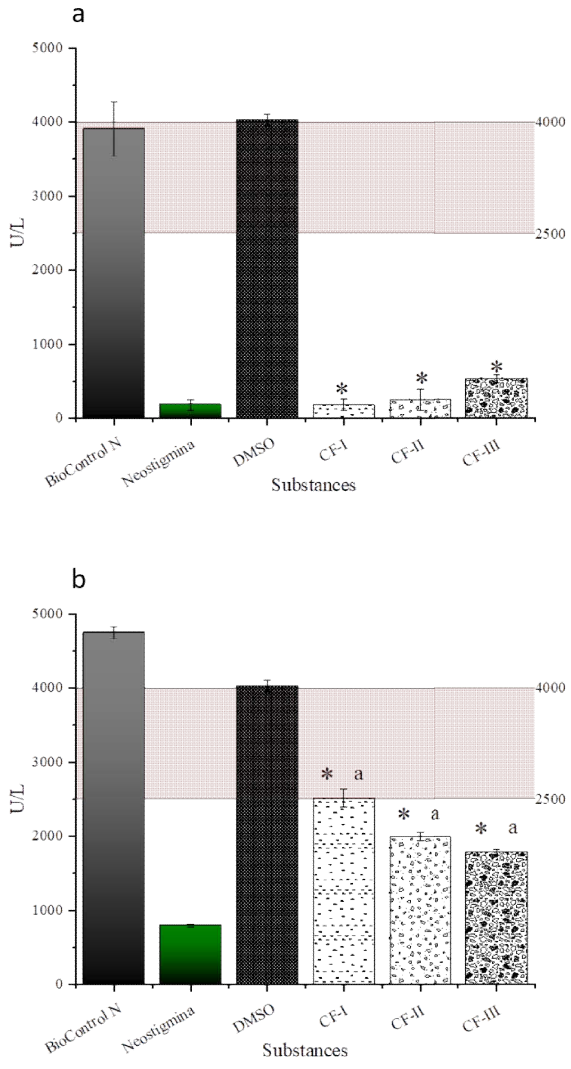

Figure 7. Plasma cholinesterase activity. *In vitro* inhibition of cholinesterase enzyme by chlorpyriphos. a: Sample extracted fromPyrinex® 480 EC; b: Chlorpyriphos from Sigma®. CF-I (4.4 mg/mL); CF-II (5.4 mg/mL); CF-III (6.4 mg/mL). Normal range is seen between 2500–4000 U/L (gray scale)

Most reports are related to*in vivo* organophosphate administration found in female rats, which inhibited plasma cholinesterase to 737 U/L (MARTY *et al*., 2012). Another study of plasma cholinesterase dosage was conducted by Richardson, Chambers, and Chambers (2001), wherein the authors used the toxic metabolites chlorpyrifos and chlorpyrifos-oxon, resulting in a potent inhibition of 52 nM. For effective cholinesterase inhibition, chlorpyriphos must be metabolized by CYP450 (ALVARENGA, 2013; TIMCHALK *et al*., 2002). Figure 7a shows that chlorpyriphos can directly inhibit cholinesterase enzyme *in vitro*.Notably, there was potent inhibition of the enzyme at three different dosages. Compared with the positive control (neostigmine, 1 mg/mL), no statistical differencewas observed betweenthe three dosages ($p > 0.05$). In 'b', there was also a direct inhibition of chlorpyriphos in contact with the cholinesterase enzyme. The major inhibition seen in 'a' can be attributed to impurities in the extracted sample. This is an important question because people may have access to the commercial formulation during a suicide attempt or even during environmental or occupational exposure and not to theextracted pesticide. This can increase the toxicity and difficulty inhandling the intoxication. Pesticide poisoning is a major problem in rural Latin American countries (e.g., El Salvador, Nicaragua, and Peru), Asian countries (e.g., the Republic of Korea and Thailand), and Portugal, notably among women (AJDACIC-GROSS *et al*., 2008).

CONCLUSION

The applied procedures were adequate, rapid, and cost-effectivein isolating the active ingredient, chlorpyriphos,from commercial Pyrinexas well as from biological samples, as confirmed by TLC and cholinesterase inhibitory activity. Both analyses are complementary for use incases of intoxication.

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